Multiple Decay Rates of Heterogeneous Nuclear RNA in HeLa Cells†

Ronald C. Herman* and Sheldon Penman

ABSTRACT: A pulse-label and chase procedure is used to measure the rates of decay of heterogeneous nuclear RNA (hnRNA) in HeLa cells. Pretreatment of the cells with glucosamine, to reduce the size of the intracellular UTP pool, makes it possible to obtain a rapid and efficient chase of the radioactive RNA precursor. Incorporation of [^{3}H]uridine abruptly ceases when unlabeled pyrimidines are added to the growth medium. The pulse-label and chase conditions, however, do not appear to affect the rate of RNA synthesis. The decay of total HeLa hnRNA follows first-order kinetics for at least 3 h and a half-life of 70 min is estimated. The poly(A)-adjacent sequences contain two kinetic components with half-lives of 60 and 150 min. These long half-lives do not result from continuing adenylation of the poly(A)$^-$ hnRNA. Sequences adjacent to oligo(A) decay more rapidly with half-lives of 30 and 125 min. The turnover of the hnRNA subfraction eluted by ammonium sulfate (shnRNA) is also examined. Approximately 3–6% of the radioactivity incorporated into pulse-labeled hnRNA appears in poly(A)$^+$ mRNA. Pulse-labeled message continues to emerge from the nucleus for at least 2 h. Actinomycin D alters both the rate of decay of hnRNA and the appearance of mRNA in the cytoplasm.

The role of heterogeneous nuclear RNA (hnRNA) in the biosynthesis of messenger RNA (mRNA) has been studied for many years (for reviews, see Lewin, 1975a,b). Using cDNA probes, it has been shown that steady-state HeLa hnRNA contains the sequences for most cytoplasmic messages (Herman et al., 1976) and that pulse-labeled fetal liver hnRNA contains the sequence for at least the globin message (Ross, 1976). However, any model which is proposed for the processing of hnRNA into mRNA must consider both the metabolic stability of hnRNA as well as the kinetics of appearance of mRNA.

Previous investigations of the metabolic behavior of hnRNA isolated from a variety of organisms indicate that, in general, the half-life of nuclear RNA is quite short (Penman et al., 1968; Soeiro et al., 1968; Perry et al., 1974; Brandhorst and McConkey, 1974). Brandhorst and McConkey (1974) examined the turnover of nuclear RNA in mammalian cells using a continuous labeling protocol. They concluded that the total nuclear RNA decayed at a single and rapid rate with a half-life estimated to be approximately 25 min. The kinetic behavior of the poly(A) segments present at the 3′ end of about 20–30% of the hnRNA molecules was also examined. Again, a single kinetic class with a 25-min half-life was identified (Brandhorst and McConkey, 1975).

Recently, Levis and Penman (1977) described a procedure for the application of a pulse-label and chase protocol to eukaryotic cells. This method is based on the observations of Scholtissek (1971) and Wertz (1975) that D-glucosamine can be used to decrease the size of the UTP pool. After a brief pulse with high specific activity [^{3}H]uridine, it is possible to lower rapidly the specific radioactivity of the intracellular UTP pool by the addition of an excess of unlabeled uridine to the growth medium. A rapid cessation of incorporation of the radioactive precursor occurs, and it is thus possible to monitor the decay of relatively unstable RNA molecules. Using a modified version of this procedure, the metabolic stability of HeLa hnRNA has been investigated. The results presented here suggest that HeLa nuclear RNA does not turn over at a single, rapid rate. Rather, it is shown that HeLa hnRNA contains several distinct components with very different metabolic stabilities.

Materials and Methods

(a) *Cell Culture.* HeLa S3 cells were grown in suspension culture at 37 °C in Dulbecco's medium supplemented with 7% horse serum (Microbiological Associates Inc.). Density was maintained at 4×10^5 cells/mL.

(b) *Uridine–Glucosamine Pulse-Label and Chase Procedure.* Suspension cultures of HeLa cells growing exponentially in Dulbecco's medium were concentrated fivefold by gentle centrifugation at room temperature and resuspension in a prewarmed mixture of 50% conditioned and 50% fresh medium. The cells were treated with actinomycin D (Calbiochem) at 0.04 μg/mL for 30 min at 37 °C, and then carrier-free [^{32}P]orthophosphate (New England Nuclear) was added to a concentration of 4 μCi/mL. After labeling for 2 h, D-glucosamine (Kodak) (adjusted to pH 7.4) was added to a final concentration of either 5 or 20 mM and incubation continued for an additional hour. The cells were then pulse labeled for 15 min with [5-^{3}H]uridine (specific activity 27 Ci/mmol; New England Nuclear) at 40 μCi/mL. The chase was initiated by a fivefold dilution of the culture with warm 50% conditioned medium containing [^{32}P]orthophosphate at 4 μCi/mL, 5 mM unlabeled uridine, 5 mM cytidine, either 5 or 20 mM glucosamine, and actinomycin at 0.04 μg/mL. Where indicated, the cells were not concentrated prior to labeling and were chased without subsequent dilution. 5-Fluorouridine was used at 5 μg/mL. For actinomycin chases, prelabeling with ^{32}P was omitted. Actinomycin was added to a final concentration of 5 μg/mL together with the other components of the uridine–glucosamine chase after a 15-min pulse label. When used, cordycepin was added at the beginning of the chase to a final concentration of 20 μg/mL.

(c) *Cell Fractionation.* All steps of the fractionation procedure were carried out at 4 °C. At each time point, a portion of the culture was removed and poured over crushed frozen Earle's saline. The cells were pelleted at 1000 rpm for 3 min, washed twice with cold Earle's saline, and then fractionated

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into nuclei and cytoplasm as described previously (Herman et al., 1976). Cytoplasmic fractions were made 0.1 M NaCl, 0.01 M EDTA[1] (pH 7.4), and 0.5% $NaDodSO_4$ and then extracted as described below. Nuclei were washed with detergent (2 parts 10% Tween 40 and 1 part 10% sodium deoxycholate) and then pelleted at 600 rpm for 3 min. The nuclear pellet was gently resuspended in 0.4 M $(NH_4)_2SO_4$–0.03 M Tris (pH 7.4) by vortexing. Chromatin was pelleted at 27 000*g* for 15 min. This chromatin pellet was used as the source of the bulk hnRNA (90%) and the supernatant as the source of the shnRNA (10%) (Price et al., 1974). Where indicated, the ammonium sulfate step was omitted and the nuclei were fractionated into nucleoplasm and nucleoli by the high salt sucrose gradient technique of Penman et al. (1968).

(d) *RNA Extraction.* The chromatin pellet was resuspended in HSB buffer [0.5 M NaCl–0.05 M $MgCl_2$–0.01 M Tris (pH 7.4)]. Pancreatic DNase (electrophoretically pure; Worthington) was added to 50 μg/mL and digestion carried out for 30 s. Two volumes of absolute ethanol was added to precipitate the nucleic acids. The ammonium sulfate supernatant was diluted fourfold with H_2O and then 2 volumes of absolute ethanol was added. RNA precipitates were spun down at 20 000*g* for 15 min and resuspended in $NaDodSO_4$ buffer [0.1 M NaCl–0.01 M Tris (pH 7.4)–0.005 M EDTA–0.5% $NaDodSO_4$]. Nuclear and cytoplasmic fractions were digested with proteinase K (250 μg/mL; EM Labs) for 20 min at room temperature. RNA was extracted by the phenol–chloroform procedure (Singer and Penman, 1973). Purified nuclear RNA was DNase treated as described previously (Herman et al., 1976).

(e). *Alkaline Cleavage and Poly(U)-Sepharose Chromatography.* hnRNA was dissolved in 4 vol of $NaDodSO_4$ buffer (0.1% $NaDodSO_4$) and placed at 0 °C. One volume of 1 N NaOH was added and the sample maintained at 0 °C for 15 min. To neutralize, 1 vol of 1 N HCl and 0.5 vol of 1 M Hepes (pH 7.4) were added. Nuclear RNA was ethanol precipitated and resuspended in 0.4 M NETS (Molloy et al., 1974). Poly(U)-Sepharose (Pharmacia) chromatography was carried out as previously described (Herman et al., 1976; Molloy et al., 1974). Oligo(A)-containing fragments were eluted with 5% formamide–ETS (Molloy et al., 1974) and poly(A)-containing fragments with 90% formamide–ETS.

(f) *Oligo(dT)-Cellulose Chromatography.* Poly(A)$^+$ mRNA and poly(A)$^+$ shnRNA were isolated by oligo(dT)-cellulose (Collaborative Research) chromatography as described by Singer and Penman (1973).

(g) *Gel Electrophoresis.* Portions of the poly(A)$^-$ cytoplasms [oligo(dT)-cellulose wash fractions] were analyzed by electrophoresis on 5-cm 10% polyacrylamide gels as described by Levis et al. (1977). The gels were scanned at 260 nm with a Joyce-Loebl UV scanner and then sliced into 2-mm pieces. Each slice was hydrolyzed overnight in NH_4OH and counted in a liquid scintillation counter. Specific activities were estimated by dividing the radioactivity in 4S and 5S RNA by the optical density of the corresponding 4S peak.

[1] Abbreviations used: hnRNA, heterogeneous nuclear RNA; shnRNA, small heterogeneous nuclear RNA subfraction; mRNA, messenger RNA; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; HSB buffer, 0.5 M NaCl–0.05 M $MgCl_2$–0.01 M Tris (pH 7.4); $NaDodSO_4$ buffer, 0.1 M NaCl–0.01 M Tris (pH 7.4)–0.005 M EDTA–0.5% $NaDodSO_4$; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; UV, ultraviolet; NETS, 0.4 M NaCl–0.01 M Tris (pH 7.4)–0.01 M EDTA–0.2% $NaDodSO_4$; ETS, 0.01 M EDTA–0.01 M Tris (pH 7.4)–0.2% $NaDodSO_4$.

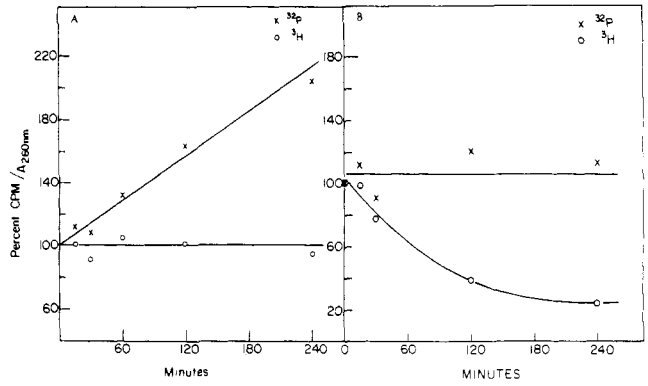

FIGURE 1: Uridine–glucosamine chase. Cells were concentrated fivefold and pretreated for 30 min with actinomycin D (0.04 μg/mL). They were then labeled for 3 h with [^{32}P]orthophosphate (4 μCi/mL); 20 mM glucosamine was added during the third hour of labeling. After a 15-min pulse with [^{3}H]uridine (40 μCi/mL), the label was chased as described in Materials and Methods. (A) Portions of the poly(A)$^-$ cytoplasms were analyzed by electrophoresis on 10% polyacrylamide gels. After scanning at 260 nm, the gels were sliced into 2-mm pieces and counted. Radioactivity in the 4S and 5S region was summed and divided by the optical density of the 4S peak. These ratios have been normalized to the zero-time (of chase) value. At the start of the chase, 19 000 cpm of [^{3}H]uridine and 3670 cpm of [^{32}P]orthophosphate were incorporated into 4S and 5S RNA. (B) The radioactivity in total hnRNA at each time point has been divided by the optical density of the cytoplasmic RNA at that time and normalized to the zero-time value: (X) ^{32}P; (O) ^{3}H.

Results

Rationale. In order to establish the utility of the glucosamine pulse-label and chase technique, it must first be demonstrated that (a) a rapid and efficient chase of the radioactive RNA precursor is obtained, and (b) that the conditions of the pulse label and chase do not adversely affect the normal metabolic behavior of the RNA. The following experimental procedure, modified where indicated, is used for the kinetic experiments described here. HeLa cell cultures are treated with a low concentration of actinomycin D to suppress nucleolar RNA synthesis (Penman et al., 1968). They are then grown for 3 h with low specific activity [^{32}P]orthophosphate in order to attain steady-state, continuous labeling of the hnRNA. Glucosamine is added to the medium at the beginning of the third hour of ^{32}P labeling. The cells are pulse labeled for 15 min with [^{3}H]uridine and the chase is subsequently initiated by adding an excess of unlabeled uridine and cytidine to the medium (see Materials and Methods).

The rate of labeling of tRNA has previously been used to monitor the specific activity of the intracellular UTP pool and therefore to determine the efficiency of a pulse-label and chase procedure (Levis and Penman, 1977). The specific activity of tRNA isolated from HeLa cells after a 15-min pulse label and uridine–glucosamine chase is shown in Figure 1A. An almost instantaneous cessation of incorporation of [^{3}H]uridine into 4S (and 5S) RNA occurs under these conditions. There is no apparent increase in specific activity of the tRNA either at the earliest time examined or at 4 h after the start of the chase. Thus, the uridine–glucosamine chase procedure appears to be quite effective in HeLa cells and can therefore be used to examine the turnover of relatively unstable RNA molecules. The cessation of incorporation of [^{3}H]uridine is probably caused by a large dilution of the pool specific activity upon the addition of an excess of unlabeled uridine at the beginning of the chase rather than by an inhibition of RNA synthesis. The incorporation of [^{32}P]orthophosphate into tRNA increases linearly

TABLE I: hnRNA Synthesis after Glucosamine Treatment.[a]

Time (min)	Control (cpm)	+ Glucosamine (cpm)	% control
30	11 863	11 046	93
60	30 531	34 554	113
120	58 594	46 068	79
240	95 476	91 781	96

[a] Cells were concentrated fivefold and pretreated for 1 h at 37 °C with actinomycin (0.04 μg/mL) and glucosamine (20 mM). Chase conditions were simulated by dilution with medium containing 5 mM uridine, 5 mM cytidine, 20 mM glucosamine, 0.04 μg of actinomycin/mL, and 20 μCi of [^{32}P]orthophosphate/mL. Control cells were incubated in an identical manner but without glucosamine and were diluted with medium containing only actinomycin (0.04 μg/mL) and [^{32}P]orthophosphate (20 μCi/mL). Nuclei were isolated as described in Materials and Methods. Acid-precipitable incorporation into alkali-sensitive material (0.3 N KOH, overnight at room temperature) was determined.

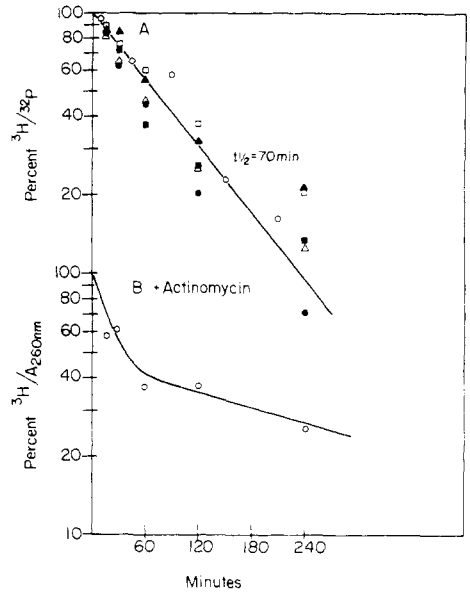

FIGURE 2: Decay of total hnRNA. Cells, 7×10^7, were pretreated and pulse labeled as described in Materials and Methods. Nuclei were prepared and ammonium sulfate treated to release the shnRNA fraction. The decay of the remaining hnRNA is shown. (A) ^{3}H cpm have been normalized to the ^{32}P cpm present in each sample and expressed as a percentage of the zero-time value: (△, ○, and □) fivefold concentrated cells pretreated with 0.04 μg of actinomycin/mL and 20 mM glucosamine. (Different symbols represent different experiments.) (▲) Fivefold concentrated cells pretreated with actinomycin and 5 mM glucosamine; (●) unconcentrated cells pretreated with actinomycin and 5 mM glucosamine; (■) nucleoplasmic RNA from fivefold concentrated cells pretreated with 5-fluorouridine (5 μg/mL) and 20 mM glucosamine. (B) Actinomycin (5 μg/mL) added at the beginning of the chase. ^{3}H cpm at each time point has been normalized to the optical density of the cytoplasmic RNA at that time and expressed as a percentage of the zero-time value.

during the chase period (Figure 1A) at approximately the same rate as prior to the addition of glucosamine.

Evidence that hnRNA synthesis continues normally in glucosamine-treated cells is shown in Table I. Cells are treated for 1 h with glucosamine in the presence of a low concentration of actinomycin. Chase conditions (see Materials and Methods) are simulated by the addition of unlabeled uridine and cytidine to the growth medium, and the incorporation of [^{32}P]orthophosphate into alkali-sensitive nuclear material is subsequently measured. As shown in Table I, the incorporation of [^{32}P]-orthophosphate into nuclear RNA in the glucosamine-treated cells is almost identical with that in control, untreated cells for at least 4 h. These results suggest that the rate of hnRNA synthesis is not significantly altered by the conditions employed for the pulse label and chase and that the rate of hnRNA turnover is also probably not affected.

Decay of Total hnRNA. Cells are pulse labeled for 15 min with [^{3}H]uridine and chased for up to 4 h. The nuclei are prepared as described in Materials and Methods and then treated with ammonium sulfate (Price et al., 1974; Herman et al., 1976). Ten percent of the hnRNA is eluted by exposure to the high ionic strength. The eluted RNA (shnRNA) has certain unique properties and its metabolic behavior will be analyzed separately. Since steady-state, continuous labeling of the hnRNA with ^{32}P is obtained under the conditions used, as indicated by the constancy of ^{32}P radioactivity during the chase (Figure 1B), RNA recoveries have been normalized to the ^{32}P cpm present in each sample.

Figure 2A shows a semilogarithmic plot of the turnover of [^{3}H]uridine pulse-labeled hnRNA. The hnRNA appears to decay at a uniform rate for at least 3 h with a half-life estimated to be approximately 70 min. There is no significant deviation from these simple first-order kinetics for at least 3 half-lives. The half-life is significantly longer than that previously estimated for mammalian hnRNA using mouse L cells (Brandhorst and McConkey, 1974). The data in Figure 2A do not, however, rule out the existence of a second, more stable, hnRNA component.

Also shown in Figure 2A are the results of several experiments employing a variety of chase conditions. The half-life of the hnRNA is unaltered if glucosamine is used at a concentration of only 5 mM during a pulse label and chase of either concentrated or unconcentrated cells (see Materials and Methods). The low concentration of actinomycin used to preferentially suppress the synthesis of nucleolar RNA (Penman et al., 1968) also does not affect the stability of the hnRNA. This is suggested by experiments using the uridine analogue, 5-fluorouridine, instead of the low concentration of actinomycin to prevent ribosomal RNA synthesis. 5-Fluorouridine, unlike actinomycin, does not inhibit the transcription of the 45S nucleolar RNA precursor, but blocks its processing into the mature rRNA species. In this experiment, the ammonium sulfate step is omitted and the nuclei are fractionated into nucleoplasm and nucleoli in order to remove the remnant nucleolar RNA (Penman et al., 1968). The decay rate of the purified nucleoplasmic RNA is shown in Figure 2A and its half-life is also estimated to be approximately 70 min.

The results presented above can be compared with the decay of hnRNA in the presence of a high concentration (5 μg/mL) of actinomycin D. This drug, added at the beginning of a uridine–glucosamine chase, markedly alters the turnover of hnRNA. Two kinetic components are observed with half-lives similar to those previously identified by Penman et al. (1968) (Figure 2B). These authors suggested that the long-lived component results from drug-induced alterations of hnRNA metabolism when continued transcription is blocked. The results obtained with the uridine–glucosamine chase support this conclusion.

Turnover of Poly(A)- and Oligo(A)-Adjacent Sequences. About 20–30% of the pulse-labeled hnRNA in HeLa cells possesses a poly(A) segment at the 3′ end of the molecule which is about 200 AMP residues in length (Derman and Darnell, 1974). A number of affinity chromatography techniques have been developed (Singer and Penman, 1973; Molloy et al., 1974)

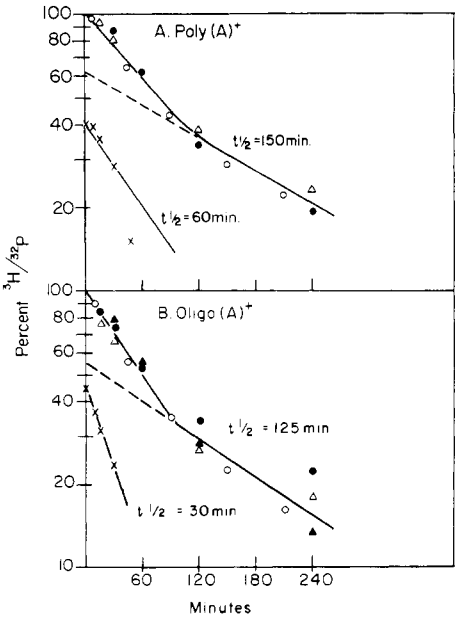

FIGURE 3: Decay of hnRNA fragments. Cells, 7×10^7, were treated as outlined in the legend to Figure 1. The isolated nuclei were exposed to ammonium sulfate to release the shnRNA fraction. The remaining hnRNA was purified and alkaline cleaved as described in Materials and Methods. ^3H cpm have been normalized to the ^{32}P cpm and expressed as a percentage of the zero-time value. (A) (●) Poly(A)-containing fragments were eluted from poly(U)-Sepharose with 90% formamide. (△ and ○) Cordycepin (20 μg/mL) was added at the beginning of the chase. (Different symbols represent different experiments.) (X) A graphical correction procedure was used to resolve the two decay rates. (B) Oligo(A)-containing fragments were eluted from poly(U)-Sepharose with 5% formamide. Data have been treated as in A. (● and ▲) Glucosamine chase; (△ and ○) cordycepin (20 μg/mL) added at beginning of chase.

to isolate the poly(A)-containing molecules. However, native poly(A)$^+$ HeLa hnRNA does not bind very efficiently to poly(U)-Sepharose, perhaps because of the large size and secondary structure of these molecules (Molloy et al., 1974; Herman, unpublished observations). A brief exposure of the large nuclear RNA to alkaline pH causes the cleavage of the molecules into smaller fragments and those containing poly(A) now bind efficiently (Molloy et al., 1974; Herman et al., 1976). Using this technique, poly(A)-containing fragments can be isolated and their kinetic behavior analyzed. Differences in the size of the fragments generated by random alkaline hydrolysis of the large molecules are normalized by the ^{32}P cpm present in each sample. Nuclear RNA also contains short internal stretches of AMP residues [oligo(A)] which, unlike the longer 3′ poly(A), are transcribed from the DNA. Oligo(A)-containing fragments are removed before the kinetic behavior of the poly(A)$^+$ fragments is analyzed. The oligo(A)-containing fragments are eluted from poly(U)-Sepharose with 5% formamide while the poly(A)-containing fragments are eluted with 90% formamide (Molloy et al., 1974; Herman et al., 1976).

The turnover of the poly(A)$^+$ fragments eluted from poly(U)-Sepharose with 90% formamide is shown in Figure 3A. The decay rate clearly deviates from simple first-order kinetics at later times. The data are interpreted to indicate the presence of two kinetic components with different rates of turnover. By applying a graphical correction procedure to resolve the two decay rates, half-lives of 60 and 150 min are obtained. These components comprise 40 and 60%, respectively, of the pulse-labeled polyadenylated hnRNA.

The relatively long half-lives observed for these components could result from continuing polyadenylation of the poly(A)$^-$ hnRNA molecules. The addition of cordycepin (3′-deoxyadenosine) at the beginning of the uridine–glucosamine chase to block further polyadenylation, however, does not alter the observed half-lives (Figure 3A). This suggests that continuing adenylation is not responsible for the relatively long half-lives that are observed.

TABLE II: Half-Lives of shnRNA.

	Component $t_{1/2}$ (min)		Proportions
	1	2	(%)
Fraction			
Total	25	120	45:55
Total[a]	30	140	45:55
Poly(A)$^+$ [a]	35	220	35:65

[a]Cordycepin, 20 μg/mL, was added at beginning of chase.

The metabolic behavior of the oligo(A)-containing portion of the hnRNA has also been examined. Oligo(A) is transcribed from the DNA and is thought to be contained only in hnRNA molecules which are not polyadenylated (Nakazato and Edmonds, 1974). Consistent with this notion is the observation that oligo(A)-adjacent hnRNA sequences have half-lives which are distinct from those of the poly(A)$^+$ fragments. Oligo(A)$^+$ fragments with half-lives of 30 and 125 min have been identified and their stability is also unaffected by cordycepin (Figure 3B).

Kinetic Behavior of Ammonium Sulfate Eluted Fraction (shnRNA). The subfraction of hnRNA which is eluted by ammonium sulfate (shnRNA) contains about 10% of the pulse-labeled nuclear material (Price et al., 1974); approximately 20% of this RNA is polyadenylated (unpublished observation). The kinetics of decay of the eluted shnRNA are distinct from those of the remaining 90% of the hnRNA. The shnRNA appears to be composed mainly of two kinetic components which are present in approximately equal proportions after a 15-min pulse label (Table II). Their half-lives are estimated to be about 25 and 120 min.

In the presence of cordycepin, the major shnRNA components decay at essentially unaltered rates (Table II). Using cordycepin to block further adenylation during the chase, the decay of the minor, poly(A)$^+$ shnRNA components has been examined. The poly(A)$^+$ shnRNA turns over somewhat more slowly, with half-lives of 35 and 220 min. The long half-life must, however, be viewed with some caution since, unlike the bulk of the hnRNA, the shnRNA fraction may be contaminated with some polyadenylated cytoplasmic species (Herman et al., 1976). Also, a direct effect of cordycepin on these decay rates has not been ruled out.

Formation of mRNA. The observed rates of decay of hnRNA are the net results of simple turnover (degradation) and conversion into cytoplasmic mRNA. The efficiency of the uridine–glucosamine chase procedure makes it possible to determine the kinetics of appearance of mRNA in the cytoplasm. Figure 4A shows the emergence of poly(A)$^+$ mRNA into the cytoplasm during the uridine–glucosamine chase. The amount of [^3H]uridine labeled poly(A)$^+$ mRNA in the cytoplasm increases for 2 h. In various experiments, 3–6% of the ^3H cpm incorporated into total nuclear RNA appears in poly(A)$^+$ mRNA during the first 2 h of the chase. At later times there is a decrease in the amount of pulse-labeled mRNA presumably because of the decay of the short-lived messenger

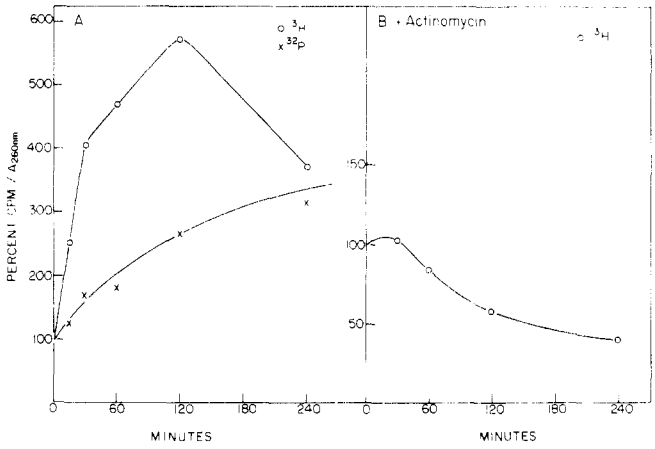

FIGURE 4: Appearance of poly(A)$^+$ mRNA. (A) Cells were pretreated and pulse labeled as outlined in the legend to Figure 1. Poly(A)$^+$ mRNA was isolated by oligo(dT)-cellulose chromatography. Radioactivity present at each time point has been divided by the optical density of the total cytoplasm at that time and expressed as a percentage of the zero-time value. (O) ^3H; (X) ^{32}P. (B) Cells were treated as in A and actinomycin (5 μg/mL) was added at the beginning of the uridine–glucosamine chase.

RNA whose half-life is estimated to be less than 2 h (Puckett et al., 1975; A. Spradling, personal communication). While pulse-labeled mRNA may continue to emerge for more than 2 h, the rate of cytoplasmic RNA turnover probably exceeds the rate of emergence from the nucleus after 2 h. In contrast, the amount of poly(A)$^+$ mRNA labeled with ^{32}P increases throughout the 4-h chase period, indicating that messenger RNA formation is uninterrupted during the chase.

As mentioned above, the kinetic behavior of hnRNA is markedly affected by high concentrations of actinomycin D. Figure 4B shows that the kinetics of emergence of poly(A)$^+$ mRNA during a uridine–glucosamine chase are also altered by the presence of actinomycin. The amount of labeled poly(A)$^+$ mRNA in the cytoplasm increases for only approximately 30 min and then slowly declines. [This emergence profile is virtually identical with that previously obtained by Penman et al. (1968).] The high concentration of actinomycin not only alters the rate of turnover of hnRNA, but blocks its conversion into mRNA.

Discussion

The uridine–glucosamine pulse label and chase procedure makes it possible to determine directly the rates of decay of mammalian nuclear RNA. These experiments do not suffer from the uncertainties that are introduced when drugs are used to block transcription (Penman et al., 1968; Scherrer et al., 1970), and they are also more sensitive than experiments using the approach to equilibrium method (Brandhorst and McConkey, 1974; Perry et al., 1974). The rate of incorporation of [^{32}P]orthophosphate into hnRNA and tRNA is constant and equal to that of control cells throughout the procedure. This suggests that RNA synthesis is normal in the glucosamine-treated cells. It is unlikely that a change in the rate of turnover of hnRNA could occur in these cells without a compensating change in either the rate of incorporation or in the amount of RNA synthesized. Furthermore, the observed rates of decay are unaltered by a variety of chase conditions.

Perhaps the most significant observation made during this study is that HeLa nuclear RNA is heterogeneous in metabolic stability as well as in size. Levis and Penman (1977) have reported that hnRNA from cultured *Drosophila* cells also contains several distinct kinetic components. Some of the poly(A)-adjacent sequences in HeLa cells decay at approximately the same rate as does the majority of the hnRNA. However, a significant portion of the poly(A)$^+$ fragments decays more slowly. Two rates of decay are also observed for the oligo(A)-containing fragments. About 40% of these turn over quite rapidly (30 min half-life), while the remainder decay more slowly. Since the long-lived poly(A)- and oligo(A)-containing molecules constitute only a small fraction of the total hnRNA, distinct kinetic components are not resolved when the population as a whole is examined. Of course, since only fragments of the poly(A)- and oligo(A)-containing molecules have been examined, it is not known whether the observed half-lives reflect the turnover of whole molecules.

Previous estimates of the rate of turnover of mammalian hnRNA, using mouse L cells, suggest that hnRNA decays at a uniform rate with a half-life of about 25 min (Brandhorst and McConkey, 1974). As is shown here for HeLa hnRNA, the initial rate of decay of poly(A)$^+$ hnRNA in mouse cells is similar to that of the total nuclear RNA. However, these investigators do not detect a second more stable poly(A)$^+$ hnRNA component in L cells. The apparent differences between the half-lives of mouse and human hnRNA may be due to differences between the cells themselves, rather than in methodology. Mouse L cells grow with a doubling time of 12–14 h (Brandhorst and McConkey, 1974), while HeLa cells double only in 24 h. Furthermore, the rates of decay of the long-lived messages in these cells can be correlated with cellular growth rates. The half-life of the long-lived mouse messenger RNA is estimated to be about 9 h (Abelson et al., 1974) while the half-life of the long-lived component of HeLa mRNA is about 21 h (Singer and Penman 1973). Therefore, it is likely that differences may also exist between the half-lives of hnRNA in these two species.

The significance of a long-lived poly(A)$^+$ hnRNA population is unclear at the present time. It is unlikely that these relatively stable molecules are merely cytoplasmic messages which contaminate the HeLa nuclear RNA preparations; the fractionation procedure used here has previously been shown by rigorous criteria to yield hnRNA virtually free of cytoplasmic contamination (Herman et al., 1976). The continued export of poly(A)$^+$ mRNA from the HeLa nucleus for at least 2 h suggests that some of these long-lived molecules may, in fact, be processed into mRNA.

The wide variations in stability exhibited by the different subfractions of hnRNA may be indicative of different fates or functions in the nucleus. Because only a very small portion of the newly synthesized hnRNA is converted into mRNA, molecules with relatively long half-lives may serve another function. It will be shown elsewhere that the steady-state hnRNA, which is composed primarily of the more stable molecules, is associated with a component of the nuclear ultrastructure and thus may play a role in nuclear organization (Herman, Hahnfeld, and Penman, in preparation).

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Effect of Proteolysis on Transcriptional Fidelity of Reconstituted Chromatin†

Robert A. Gadski and Chi-Bom Chae*

ABSTRACT: The effect of proteolysis on the transcriptional properties of reconstituted rat liver chromatin was studied. Within the sensitivity of currently available methods, proteolysis of chromosomal proteins by chromatin-bound protease during chromatin reconstitution has no apparent effect on: (1) number of initiation sites, (2) proportion of reiterating and unique sequences of DNA transcribed, (3) size of the RNA transcribed, and (4) transcription of DNA sequences complementary to poly(A) containing messenger RNA.

Recently there have been several reports on the transcriptional fidelity of reconstituted chromatin (Gilmour and Paul, 1970; Paul et al., 1973; Spelsberg and Hnilica, 1970; Barrett et al., 1974; Stein et al., 1975). For example, both the transcriptions of globin and histone gene sequences from reconstituted reticulocyte chromatin (Paul et al., 1973; Barrett et al., 1974) and HeLa cell chromatin at S phase (Stein et al., 1975) have been demonstrated. Studies on the mechanism of chromatin reconstitution would therefore provide important information toward elucidating the role of chromosomal proteins in gene regulation.

We have found, however, that chromosomal proteins are considerably degraded during chromatin reconstitution by a chromatin-bound protease (Chae, 1975; Chae and Carter, 1974; Chae et al., 1975). Therefore, the effect that proteolysis has on the transcriptional properties of reconstituted chromatin, and the possibility that this effect could be altered by an inhibitor of protease, was of great interest. Rat liver chromatin was chosen for these studies since the patterns of protein degradation (Carter and Chae, 1976) and the protease responsible for the degradation (Carter et al., 1976) have both been studied in some detail. Also, only the transcription of redundant sequences of DNA from native and reconstituted liver chromatin was studied in earlier work (Gilmour and Paul, 1970; Spelsberg and Hnilica, 1970).

Experimental Section

Preparation of Chromatin. Nuclei were prepared by homogenizing rat liver tissue in 0.25 M sucrose, 3 mM $MgCl_2$, and 10 mM potassium phosphate (pH 6.5). All steps were done at 4 °C unless otherwise indicated. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 700–1000*g* for 5 min, and the nuclei were suspended in 2.3 M sucrose, 3 mM $MgCl_2$, and 10 mM potassium phosphate (pH 6.5) and recentrifuged at 22 000 rpm in a Beckman 30 rotor for 45 min. The nuclear pellet was then suspended in 1% Triton X-100 containing 0.25 M sucrose, 3 mM $MgCl_2$, and 10 mM potassium 1000*g* for 5 min. Further purification of the chromatin was accomplished by the procedure of Chae (1975).

Dissociation and Reconstitution of Chromatin. Chromatin was dissociated by bringing the chromatin solution to 2 M NaCl–5 M urea and 10 mM Tris-HCl (pH 7.5). The chromatin was stirred for 4 h at 4 °C. Chromatin reconstitution was carried out by a modification of the gradient dialysis procedure of Bekhor et al. (1969). The salt concentration was reduced

† From the Department of Biochemistry and Nutrition, University of North Carolina, Chapel Hill, North Carolina 27514. *Received October 10, 1976; revised manuscript received March 2, 1977.* Supported by a grant (CA-17390) from the National Cancer Institute, National Institutes of Health, and a grant (GM-21846) from the Institute of General Medical Sciences, National Institutes of Health.

[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; cDNA, complementary deoxyribonucleic acid; SSC, standard saline-citrate (0.15 M NaCl–0.015 M sodium citrate); HnRNA, heterogeneous nuclear ribonucleic acid.